ORIGINAL ARTICLE

In vitro activity of a new echinocandin, LY303366, and comparison with fluconazole, flucytosine and amphotericin B against *Candida* species†

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Objective To investigate the in vitro activity of LY303366 (LY) against *Candida* isolates comprising nine different species and comparison with fluconazole (FLU), flucytosine (5FC) and amphotericin B (AMB).

Methods The method used was a microtitre modification of the NCCLS M27-A accepted standard using either RPMI-1640 with 2% glucose (5FC and FLU) or antibiotic medium 3 with 2% glucose (LY and AMB). The minimum inhibitory concentration (MIC) was the lowest drug concentration that reduced growth by 80% compared with the drug-free control. Minimum fungicidal concentrations (MFCs; 99% kill) were also determined for all isolates for LY and AMB.

Results Overall, 58 of 105 (55.2%) isolates were resistant to FLU (MIC $\geq$ 16 mg/L). There was no relationship between FLU and LY MICs for *C. albicans* or non-*albicans* species. For all isolates, geometric mean (GM) MIC values and ranges (in mg/L) were: LY 0.011 and $\leq$0.001–16, FLU 8.72 and $\leq$0.125–> 128, 5FC 0.393 and $\leq$0.03–> 32, AMB 0.046 and 0.008–0.125. Differences in susceptibility to LY were seen: *C. parapsilosis* (n = 12, GM 0.4 and range 0.125–16) and *C. guilliermondii* (n = 8, GM 0.46 and range 0.25–1) were both found to be significantly less susceptible to LY than all other species ($P \leq 0.05$). For all isolates, geometric mean MFC values and ranges (in mg/L) were: LY 0.032 and 0.002–16, AMB 0.143 and 0.03–2. The MFC value was the same as or only one drug dilution higher than the MIC value for 69.5% and 48.6% of isolates tested for LY and AMB, respectively. Tolerance was described in 13.3% and 5.7% of isolates for LY and AMB, respectively. A reproducibility study performed on 20% of the isolates showed that 90.5%, 100%, 95.2% and 100% of isolates retested were the same or within one well of the original MIC value for LY, FLU, 5FC and AMB, respectively.

Conclusions LY303366 shows promising antifungal activity in vitro and warrants further in vivo investigation.

Keywords LY303366, echinocandin, *Candida*, susceptibility, tolerance, fluconazole, flucytosine, amphotericin B

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INTRODUCTION

The increasing incidence of serious fungal infection is a well-recognized problem. Such infections, predominantly affecting immunocompromised individuals, require prompt and appropriate therapy. However, the choice of drugs is somewhat limited. Amphotericin B (AMB) is associated with numerous toxic side-effects, although newer less toxic formulations are now available. Fluconazole (FLU) has low toxicity and is well absorbed; however, the emergence of resistant isolates as a consequence of long-term use, particularly in HIV-positive patients, is increasing in incidence [1,2]. Hence, there is a need to develop new, safe and effective antifungal drugs. LY303366 (LY) is a semisynthetic derivative of the echinocandin class of antifungal agents, whose mode of action is the inhibition of cell wall synthesis. The drugs act specifically by inhibiting (1,3)-β-D-glucan synthase, an enzyme complex that forms glucan polymers in the fungal cell wall [3]. Not surprisingly, LY has therefore been reported to have good in vitro activity against many yeast species, including *Candida* [4–7] and *Saccharomyces* [5], but not *Cryptococcus* [8] or *Trichosporon* [6]. In vivo activity has also been described against *Candida* [9,10]. In

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addition, LY has been shown to have in vitro and in vivo activities against *Aspergillus* [6,8,11,12], and other filamentous fungi [6], *Pneumocystis carinii* [13] and *Histoplasma capsulatum* [6,14]. In this study, we directly compared the antifungal activity of LY with FLU, flucytosine (5FC) and AMB against a wide range of *Candida* isolates, including some of the more unusual species. We selected a population of isolates with a disproportionate number resistant to fluconazole. In addition, we examined the fungicidal activity and tolerance of LY and compared it directly with that of AMB.

MATERIALS AND METHODS

Organisms

Susceptibility tests were performed on 105 isolates of *Candida* belonging to nine different species. These consisted of 99 recent clinical isolates from a variety of patient types, including patients with AIDS, candidaemia and tissue-invasive disease, and six American Type Culture Collection (ATCC) isolates. Of the patient isolates, 81% were from mucocutaneous sources, 10% were hematogenous, and 9% from other sites. The group comprised 42 *C. albicans*, 13 *C. glabrata*, 12 *C. parapsilosis*, 11 *C. krusei*, 10 *C. tropicalis*, eight *C. guilliermondii* and three each of *C. lusitaniae*, *C. norvegensis* and *C. inconspicua*. In addition, a disproportionate number of isolates were resistant to FLU. Each culture was grown on Sabouraud agar at 37 °C for 48 h to ensure purity.

Antifungal agents

LY303366 (Eli Lilly & Co., Indianapolis, IN, USA), fluconazole (Pfizer, Kent, UK) and flucytosine (Roche Products Ltd, Hertfordshire, UK) were all provided in pure powder form by their respective manufacturers. Amphotericin B with desoxycholate was obtained from E. R. Squibb & Sons Ltd, Middlesex, UK.

LY was dissolved in dimethyl sulfoxide, adjusting the weight to allow for the potency of the drug, to produce a stock solution of 1280 mg/L. All other drugs were dissolved in sterile distilled water, adjusting for potency where necessary, to give stock solutions of 1280 mg/L. All drug stocks were then dispensed into aliquots and stored in glass vials, protected from the light, at −20 °C until required.

Media

The choice of media for LY was determined during a small pilot study prior to this study. Antibiotic medium 3 (pH 7.0; Difco, Surrey, UK) with 2% glucose was selected since it supported good growth of all species and gave clear-cut end-points with very little or no trailing effect. This was in contrast to various other media that included RPMI-1640 (Sigma, Dorset, UK) with 2% glucose, high resolution medium (Oxoid, Basingstoke, UK) and yeast nitrogen base (Difco, Surrey, UK) supplemented with 1% glucose. Antibiotic medium 3 with 2% glucose was also used for AMB [15]. RPMI-1640 with 2% glucose, buffered with morpholinopropanesulfonic acid (MOPS, Sigma) and adjusted to pH 7.0 was used for FLU and 5FC [16]. The entire study, including reproducibility, was performed using the same batch of each medium.

Susceptibility testing

The method used was a microtitre modification of the NCCLS M27-A accepted standard [2,16]. Final drug ranges (in mg/L) were 0.001–32 for LY, 0.125–128 for FLU, 0.03–32 for 5FC and 0.004–8 for AMB.

Yeast suspensions were prepared by suspending single colonies in sterile 0.85% saline. The turbidity of each suspension was measured on a spectrophotometer at an optical density of 490 nm. Each suspension was then adjusted, using the appropriate medium, to give a final inoculum of 1×10^3 organisms/mL. One well for each isolate was left drug-free to act as a positive control. A solvent control was included for LY ensuring that any inhibition of growth was due to drug alone. Negative controls were also included to ensure sterility of each medium. The plates were incubated in a moist chamber at 37 °C for 48 h. Sabouraud agar and blood agar plates were inoculated with 25 μL of each organism suspension to check the viable count and culture purity.

After incubation, the plates were shaken for 5 min to obtain a uniform suspension prior to reading. The growth in each well was measured by determining the optical density at 490 nm by spectrophotometer. The minimum inhibitory concentration (MIC) was taken as the lowest drug concentration that reduced the OD_{490} by 80% compared with the drug-free control.

Minimum fungicidal concentrations (MFCs) were also determined for LY and AMB. For each isolate, 100 μL was removed from the MIC well and all concentrations above it. Each aliquot was spot-inoculated onto a blood agar plate and the liquid was allowed to dry. The plate was then streaked with a sterile loop, thereby removing yeast cells from the drug source, and incubated at 37 °C for 48 h. The MFC was defined as the lowest concentration of drug at which less than two colonies grew (99% kill).

Quality control

C. albicans ATCC 90028, *C. albicans* ATCC 24433, *C. glabrata* ATCC 90030, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 and *C. tropicalis* ATCC 750 were tested to ensure quality control [17].

Reproducibility

Twenty per cent of isolates (21/105) were randomly selected and re-tested against each drug to establish the reproducibility of the method.

Statistical analysis

The differences between species were analyzed for each drug by a one-way analysis of variance with a Bonferroni correction for multiple comparisons (SPSS for Windows). For data analysis, MICs of ≤ 0.001, ≤ 0.03 or ≤ 0.125 mg/L were recorded as 0.001, 0.03 or 0.125 mg/L for LY, 5FC and FLU, respectively. Values of >32 or >128 mg/L were classed as 64 and 256 mg/L for 5FC and FLU, respectively.

RESULTS

Summaries of the in vitro susceptibility values of the 105 isolates tested are shown in Table 1. Overall 55.2% of the isolates were resistant to fluconazole (MIC $\geq$ 16 mg/L). However, no differences between LY geometric mean (GM) MIC values (mg/L) were apparent when comparing FLU-susceptible ($n = 47$,

Table 1 *In vitro* susceptibilities of 105 *Candida* isolates to LY303366, amphotericin B, fluconazole and flucytosine

Species (No. of isolates)	Antifungal agent	MIC (mg/L)			
		Geometric mean	Range	50%	90%
C. albicans (42)	LY	0.0029	≤ 0.001–0.015	0.002	0.0078
	AMB	0.05	0.015–0.06	0.06	0.06
	FLU	6.24	≤ 0.125 –> 128	8	128
	5FC	0.28	≤ 0.03 –> 32	0.25	1
C. glabrata (13)	LY	0.01	0.0039–0.03	0.0078	0.03
	AMB	0.075	0.06–0.125	0.06	0.125
	FLU	60.68	16 –> 128	32	> 128
	5FC	0.064	0.06–0.125	0.06	0.06
C. parapsilosis (12)	LY	0.4	0.125–16	0.25	1
	AMB	0.025	0.015–0.03	0.03	0.03
	FLU	0.79	0.5–2	1	1
	5FC	0.11	0.06–0.25	0.125	0.25
C. krusei (11)	LY	0.011	0.0078–0.015	0.0078	0.015
	AMB	0.084	0.06–0.125	0.06	0.125
	FLU	56.42	32–128	64	128
	5FC	9.66	4–16	8	16
C. tropicalis (10)	LY	0.006	0.002–0.015	0.0078	0.0078
	AMB	0.037	0.015–0.06	0.03	0.06
	FLU	10.56	0.5 –> 128	8	> 128
	5FC	0.81	0.06 –> 32	0.125	> 32
C. guilliermondii (8)	LY	0.46	0.25–1	0.5	1
	AMB	0.025	0.0078–0.06	0.03	0.03
	FLU	7.34	4–16	4	16
	5FC	0.072	≤ 0.03–0.125	0.06	0.125
C. lusitaniae (3)	LY	0.015	0.015	0.015	0.015
	AMB	0.048	0.03–0.06	0.03	0.06
	FLU	0.2	≤ 0.125–0.5	≤ 0.125	0.5
	5FC	6.26	0.06 –> 32	0.06	> 32
C. inconspicua (3)	LY	0.0016	≤ 0.001–0.002	≤ 0.001	0.002
	AMB	0.024	0.0078–0.06	0.0078	0.06
	FLU	32	16–64	16	64
	5FC	4	4	4	4
C. norvegensis (3)	LY	0.0024	≤ 0.001–0.0039	≤ 0.001	0.0039
	AMB	0.038	0.015–0.06	0.015	0.06
	FLU	32	32	32	32
	5FC	6.35	4–8	4	8
All isolates (105)	LY	0.011	≤ 0.001–16	0.0078	0.25
	AMB	0.046	0.0078–0.125	0.06	0.06
	FLU	8.72	≤ 0.125 –> 128	16	128
	5FC	0.393	≤ 0.03 –> 32	0.125	8

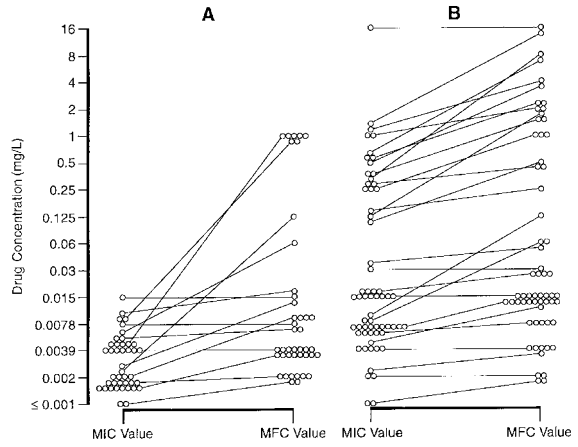

Figure 1 The relationship between MIC and MFC values for *Candida albicans* ($n = 42$) [A] and non-*albicans* species ($n = 63$) [B] against LY303366.

LY GM 0.018 and range $\leq$0.001–16) and FLU-resistant ($n = 58$, LY GM 0.007 and range $\leq$0.001–0.5) isolates.

All *C. albicans* isolates were susceptible to LY (Figure 1A) and, assuming a breakpoint of $\geq$0.125 mg/L, 20 of 63 (31.7%) of non-*albicans* isolates were resistant (Figure 1B). These isolates were all *C. parapsilosis* or *C. guilliermondii*. However, if a breakpoint of $\geq$2 mg/L is selected, only one isolate of *C. parapsilosis* (1.6%) was resistant. All isolates (*C. albicans and* non-*albicans* species) were susceptible to AMB (MIC $\leq$ 0.25 mg/L) (Figure 2).

Cidal activity was evaluated by determining whether the yeast cells were still viable after the drug source was removed. The geometric mean MFC values (and ranges) for all 105 isolates (in mg/L) were: LY 0.032 (0.002–16) (Figure 1) and AMB 0.143 (0.03–2) (Figure 2). The MFC value was the same as, or only one drug dilution higher than, the MIC value for 69.5% and 48.6% of isolates tested for LY and AMB, respectively. Using the criterion of a 10-fold increased MFC above the MIC for tolerance, 9/42 (21.4%) and 1/42 (2.4%) of *C. albicans* isolates were tolerant to LY and AMB, respectively, and 5 of 63 (7.9%) of non-*albicans* isolates for both LY and AMB.

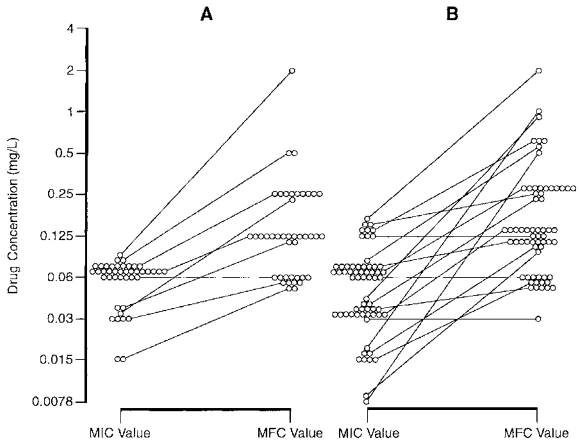

Figure 2 The relationship between MIC and MFC values for *Candida albicans* ($n = 42$) [A] and non-*albicans* species ($n = 63$) [B] against amphotericin B.

Isolates of *C. parapsilosis* ($n = 12$) and *C. guilliermondii* ($n = 8$) were significantly less susceptible to LY than all other species tested ($P \leq 0.05$). The geometric mean MICs and MFCs (and ranges) of *C. parapsilosis* for LY (in mg/L) were 0.4 (0.125–16) and 1.33 (0.25–16), respectively. For *C. guilliermondii* the same values were 0.46 (0.25–1) and 3.67 (2–16), respectively.

End-points were usually clear-cut. Overall for LY, the MIC value for 21/105 (20%) of isolates would have increased by one twofold dilution if a no-growth end-point had been used. Only one isolate (0.95%) (*C. guilliermondii*) would have had a MIC value increased by two dilutions (from 0.25 to 1 mg/L). In comparison, for AMB, 17% (18/105) of isolates would have had a MIC value increased by only one dilution if a no-growth end-point had been chosen. Seven of these isolates were *C. parapsilosis*.

In order to check the reproducibility of the method, the MIC test was repeated on 21 isolates (20%) with all four drugs. For LY, 19/21 (90.5%) isolates gave MIC results within one well (twofold) difference. All 21 isolates when re-tested against FLU and AMB produced either identical results or gave a result that differed by one twofold dilution only. Repeat testing with 5FC showed that 20/21 (95.2%) isolates gave MIC results within one well difference.

DISCUSSION

This study confirms that in vitro testing of LY is possible with reproducible end-points. It yields a range of susceptibility values and shows that, for most isolates, LY303366 is fungicidal. Based on preliminary work, we chose to use antibiotic medium 3 with 2% glucose as the test medium. It has previously been reported that the activity of LY was considerably more potent when tested in antibiotic medium 3 than in RPMI-1640 [5]. Certainly, the MIC values obtained in this study are much lower (between 10- and 60-fold lower when comparing MIC50 values for *C. albicans*) than previous studies that utilized RPMI-1640 [4–8] and are comparable to reported values using antibiotic medium 3 (without additional glucose) [5]. In addition, as we observed during preliminary work, MICs determined for LY using RPMI-1640 may be subject to a more pronounced trailing effect. This observation led to the finding that an 80% reduction in visible growth might give a better correlation with fungicidal activity [18]. However, using antibiotic medium 3 with 2% glucose, end-points with LY were usually clear-cut with very little, if any, trailing effect.

Further in vitro/in vivo comparison studies are necessary to establish which medium will provide the better correlation. This work is vital to the future employment of in vitro testing of LY as a means of predicting clinical outcome.

LY is active against all *Candida* species tested and overall at concentrations 800-fold lower than fluconazole (population selected for resistance), nearly 40-fold lower than flucytosine and fourfold lower than AMB. Compared with AMB, LY is fungicidal at 4.5-fold lower concentrations. However, LY is significantly less active against isolates of *C. parapsilosis* and *C. guilliermondii*. These results are in accord with previous findings [4,5,8]. *C. guilliermondii* is generally susceptible to all antifungal agents tested. However, resistance to AMB has been described in this species [19]. Interestingly, terbinafine is also less active against *C. parapsilosis* but in vitro resistance to fluconazole has only rarely been described in this organism [20,21]. Well-documented cases of persistent fungaemia in the face of AMB and FLU are described [21,22]. LY was active against *C. krusei*, which is intrinsically resistant to FLU [23,24] and less susceptible to AMB [23,25]. *C. inconspicua,* a rare pathogen, closely related to *C. krusei* [26], may be FLU-resistant [27] but is susceptible to LY. *C. lusitaniae* is usually susceptible to FLU and 5FC [25,28] but may be resistant to AMB [29]. It was, however, susceptible to LY in this study, as it was to AMB. Most importantly, multiple isolates of *C. albicans, C. tropicalis and C. glabrata* that were resistant to FLU were fully susceptible to LY. This is in agreement with findings reported by others [7]. As these organisms are increasing causes of candidaemia in many centres [30–32], these data are potentially clinically valuable if the in vitro results are translated into clinical efficacy.

At this stage of the drug's development, it is not possible to determine breakpoints for susceptibility or resistance. We have identified two possible MIC breakpoints ($\geq$ 0.125 mg/L and $\geq$2 mg/L) based on the distribution of values we obtained. The first is consistent with clinical outcome data for amphotericin B [33] but these have yet to be fully validated. Pharmacokinetic studies show that a single oral dose of 200 mg of LY gives plasma concentrations in excess of 156 mg/mL for more than 24 h [34]. This concentration substantially exceeds all the MIC values recorded in this study, except those of *C. parapsilosis* and *C. guilliermondii*. The drug was also well tolerated in healthy volunteers at oral doses of up to 500 mg [34]. In a rabbit model of disseminated candidiasis, LY was well tolerated at doses of up to 1.0 mg/kg/day [10]. In addition, peak plasma levels were greater than the MIC value (0.015 mg/L) of the test strain used in the model for doses as low as 0.1 mg/kg, and doses of 0.5 mg/kg sterilized all tissues, including the brain. Additional work will need to be done to establish a breakpoint.

Some isolates showed apparent tolerance. The definition of tolerance has varied from a 10- to 32-fold increased cidal concentration over the inhibitory concentration, with regard to bacteria. Using the less stringent definition of a 10-fold increase, 14 (13.3%) isolates were tolerant to LY – 21.4% of *C. albicans* and 7.9% of non-*albicans* species. Many fewer *C. albicans* isolates were tolerant to AMB (2.4%), but the same number of non-*albicans* species. Tolerance to AMB has previously been described in *C. parapsilosis* [35]. Interestingly, previous time-kill studies with LY have found that fungicidal activity was invariably observed when antibiotic medium 3 was used as the test medium, but not always with RPMI-1640 [36]. However, other studies using RPMI-1640 suggest that LY is fungicidal at concentrations at or close to the MIC value [6]. In addition, flow cytometric assays indicate that LY is so rapidly fungicidal that a 5-min exposure to the drug kills >99% of the cells [37]. In highly immunocompromised patients with life-threatening disease, or those with endocarditis or central nervous system infection, tolerance could be an undesirable characteristic. Therefore, further work is warranted on this point, given the substantial isolate-to-isolate variation.

LY303366 shows promising antifungal activity in vitro warranting further in vivo investigation. It is also now undergoing clinical trials.

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